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The effect of combined temperature-pressure treatments on pepper (*Capsicum annuum*) pectin methylesterase in model systems

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Abstract
At atmospheric pressure, purified pepper PME starts to inactivate at temperatures higher than 54°C. But when mild temperature/high-pressure treatments are applied (T≥54°C, P≤300MPa), an antagonistic effect of pressure and temperature is observed. Pressure seems to wield a protective effect on heat inactivation. Within the thermal/high-pressure domain investigated, maximal pepper PME activity in the presence of pectin was observed at 200MPa/55°C. At temperatures lower than 50°C, PME activity decreased with pressure; while at temperatures higher than 50°C, there was an enhancement of the pressure effect from 0.1-200MPa, followed by a decrease for higher pressures.

Keywords: *Capsicum annuum*; pectin methylesterase; temperature; high-pressure.

1. Introduction
Thermal processing is commonly used to increase the shelf-life of food products. Through heating pathogenic and spoilage microorganisms are killed and deteriorating enzymes can be inactivated. An increasing demand for minimally processed products that resemble fresh ones has driven the search for new mild preservation techniques, which are able to better preserve the initial quality of foods has also been intensified. Among these, high-pressure processing is gaining the most interest. During industrial processing of pectin containing food products, modifications within the pectin structure can often occur, either caused by chemical conversions, or by the action of endogenous pectin degrading enzymes, i.e. pectin methylesterase (PME). The reactions catalysed by this enzyme can influence the functional properties of foodstuffs in a positive/negative way. Insufficient PME inactivation is responsible for cloud destabilization of juices (Castaldo et al., 1996) whereas controlled enhancement of the PME catalysed reaction can improve texture of fruits and vegetables (Waldron, 2004).

The aim of this work is to study the effect of thermal and combined thermal/high-pressure treatments on the stability and activity of purified pepper PME in model systems (citrate buffer and pectin) at pH 5.6.

2. Material and methods
2.1 Extraction and purification of green bell pepper PME
Green bell peppers (*Capsicum annuum*), purchased from a local auction (Mechelen, Belgium), were cut into small pieces, frozen in liquid nitrogen and stored at −80°C until use. PME was extracted from peppers with 0.2 M Tris(hydroxymethyl)-

aminomethan buffer (i.e., Tris buffer) (pH 8.0) with 1 M NaCl, followed by purification using affinity chromatography on a NHS-Sepharose-PME-inhibitor column, according to Ly Nguyen et al. (2002). The PME fractions were pooled together and concentrated with Centricon Plus 10 (Millipore) for further analysis.

2.2 Thermal and high-pressure treatments of green bell pepper PME

For the thermal inactivation studies, the enzyme solution (citrate buffer, pH 5.6) was enclosed in glass capillaries (Hirschmann, Germany), while for the thermal activity studies the substrate-enzyme solution was put into individual pyrex tubes. The enzymatic reaction catalysed by purified pepper PME was initiated at atmospheric pressure by adding purified pepper PME to 30 mL to 0.4% (w/v) pectin solution containing 0.4 M of NaCl (citrate buffer, pH 5.6). Both types of experiments were performed in a temperature-controlled water bath, during pre-set time intervals.

Combined thermal/high-pressure treatments for both inactivation and activity studies were conducted in a multivessel high-pressure apparatus (8×8 mL) (Resato, Roden, The Netherlands). The pressure medium is a glycol-oil mixture (TR-15, Resato). To enclose both enzyme solution and the substrate-enzyme solutions, flexible microtubes of 0.3 mL were used (Elkay, Leuven, Belgium). The microtubes were placed in the pressure vessels, already equilibrated at the desired temperature. After the equilibration period, one vessel was decompressed and considered as the initial PME activity (A_0), while the remaining vessels were decompressed at pre-set time intervals.

2.3 PME activity measurement

For the inactivation studies, residual PME activity was measured by continuous recording of titration of carboxyl groups released from a pectin solution using an automatic pH-stat (Metrohm, Switzerland) and 0.01 N NaOH solution. Routine assays were performed with a 3.5 mg/mL apple pectin solution (DE 75%, 30 mL) containing 0.117 M NaCl (pH 7.0) at 22°C. The activity unit (U) of PME is defined as the amount of enzyme required to release 1 µmol of carboxyl groups per minute, under the aforementioned assay conditions. The activity of PME is proportional to the rate of NaOH consumption $(\Delta V_{NaOH} / \Delta t)$.

For the activity studies, after both thermal and thermal/high-pressure treatments, the enzymatic reaction was quenched by a heat shock (85°C, 2 min) and the release of methanol, produced during the reaction of PME on pectin as a function of time, was determined colorimetrically according to Klavons and Bennett (1986). The amount of methanol formed in a pectin solution without enzyme addition, was also determined due to the possibility of chemical hydrolysis.

2.4 Data analysis
2.4.1 Inactivation studies

Recently, Castro et al. (2004) have shown that *Capsicum annuum* has two different PME isozymes, i.e. a labile and a stable fraction. According to its different behaviour towards temperature and/or pressure, two kinetic models could be applied to describe its inactivation behaviour. When both isozymes inactivate according to a first order kinetic model, a biphasic model was considered [Eq. (1)]:

$$A = A_L \exp(-k_L \cdot t) + A_S \exp(-k_S \cdot t) \tag{1}$$

where A_L, k_L and A_S, k_S denote the activity and the inactivation rate constants (k-values) for labile and stable fractions, respectively. When only the labile fraction

inactivates, whereas the activity of the stable fraction does not change with time, a non-zero residual activity after prolonged thermal and/or pressure treatment is obtained (A_∞), described by a fractional conversion model [Eq. (2)]:

$$A = A_\infty + (A_0 - A_\infty)\exp(-k \cdot t) \tag{2}$$

2.4.2 Activity studies

The reaction catalysed by purified pepper PME was followed by measuring the release of methanol during thermal or combined pressure/temperature treatments. PME activity [µg MeOH mL^{-1} (pectin solution) min^{-1}] was estimated from the initial linear part of the curve obtained by plotting the amount of methanol formed as a function of time (V_0). Due to variations of the diluted solution of purified pepper PME daily prepared, it was necessary to divide V_0 by PME activity [µmol acid mL^{-1} (pectin solution) min^{-1}], as determined under standard assay conditions (measured titrimetrically at 22.0°C, pH 7.0). The normalized PME activity is denoted as V_0'.

The Arrhenius equation was used for determining the temperature dependence of both k-values and V_0' at given pressure, expressed by the activation energy (E_a, kJmol^{-1}) [Eqs. (3) and (4)]; while the Eyring equation was used for determining the pressure dependence of both k-values and V_0' at given temperature, expressed by the activation volume (V_a, cm^{3}mol^{-1}) [Eqs. (5) and (6)].

$$k = k_{ref} \cdot \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \tag{3}$$

$$k = k_{ref} \cdot \exp\left[-\frac{V_a}{RT}(P - P_{ref})\right] \tag{5}$$

$$V_0' = V_{0,ref}' \cdot \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \tag{4}$$

$$V_0' = V_{0,ref}' \cdot \exp\left[-\frac{V_a}{RT}(P - P_{ref})\right] \tag{6}$$

Where T and T_{ref} are the absolute temperature (K) and the reference temperature (K), respectively; P and P_{ref} are the pressure and the reference pressure, respectively; k_{ref} is the inactivation rate constant at T_{ref} or P_{ref}; $V_{0,ref}'$ is the Initial rate of pepper-PME-catalysed methanol formation at T_{ref} or P_{ref}; and R (8.314 Jmol^{-1}K^{-1}) is the universal gas constant.

3. Results and discussion
3.1 Inactivation studies of purified pepper PME

The inactivation of purified pepper PME at pH 5.6 was studied under thermal and thermal/high-pressure treatments, within the range of 10-64°C and 0.1-800 MPa. The thermostable fraction represented 20% of the total activity (data not shown), which is less than the value (57-62%) found during the inactivation study of purified pepper PME at pH 7.5 (Castro et al., 2004). It is well known that the amount of thermostable PME fraction can vary, among other factors, with pH, cultivars degree of maturity and experimental differences in the procedure (Wicker, 1992; Snir et al., 1996). No complete inactivation of purified pepper PME was attained when the enzyme solution was submitted to different combinations of pressure/temperature.

Table 1: Kinetic parameters estimates (k-values, $10^2 \cdot min^{-1}$) for combined pressure-temperature inactivation of labile fraction of purified pepper PME in citrate buffer, pH 5.6 (Pressure: MPa; Temperature: °C).

P/T	10	25	30	40	50	54	56	60	62	64	E_a (kJmol⁻¹)
0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.22±0.26[a]	15.35±0.71[a]	37.41±1.12[a]	79.22±3.27[a]	371.6±7.4 (1.00)
100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.95±0.16[a]	8.28±1.19[a]	11.38±0.74[b]	15.63±5.48[b]	193.6±17.2 (0.96)
200	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.04±0.14[a]	4.17±1.05[a]	n.d.	8.33±2.64[b]	162.3±0.4 (1.00)
300	n.d.	n.d.	n.d.	n.d.	n.d.	1.05±0.38[a]	1.82±0.77[a]	2.83±1.88[b]	2.94±0.62[b]	6.63±1.71[b]	143.3±25.8 (0.91)
400	n.d.	n.d.	n.d.	n.d.	1.01±0.51[a]	2.04±0.53[a]	2.86±0.48[a]	7.71±2.02[b]	7.95±3.87[b]	10.79±2.70[b]	158.7±10.7 (0.98)
500	n.d.	n.d.	n.d.	0.78±0.20[a]	2.05±0.29[a]	3.52±0.80[b]	3.73±0.70[b]	11.61±1.62[b]	15.38±8.22[b]	n.d.	111.6±13.3 (0.95)
600	0.56±0.07[a]	2.22±0.36[a]	2.81±0.31[a]	3.85±1.07[b]	6.38±1.63[b]	8.84±0.80[b]	13.49±4.69[b]	n.d.	n.d.	n.d.	47.3±3.8 (0.97)
700	2.19±0.72[a]	7.14±1.94[a]	7.66±1.95[a]	17.66±1.15[b]	20.72±1.84[b]	n.d.	30.69±4.97[b]	n.d.	n.d.	n.d.	42.4±3.8 (0.97)
800	7.42±1.07[b]	17.16±3.71[b]	21.42±2.69[b]	39.40±2.81[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	40.5±1.7 (1.00)
V_a (cm³mol⁻¹)	-30.33±0.93 (1.00)	-25.35±2.08 (0.99)	-25.60±0.19 (1.00)	-34.60±3.48 (0.98)	-27.40±2.13 (0.99)	-19.01±1.09 (0.99)	-19.71±2.88 (0.94)	-19.55±4.74 (0.94)	-19.60±4.69 (0.95)		

n.d.: not determined; [a] fractional conversion model; [b] biphasic model; the values after the ± correspond to the standard error; and R²-values are between parenthesis.

Under mild-heat and high-pressure conditions, only the labile fraction was inactivated, whereas the activity of the stable fraction remained unchanged with respect to time. Therefore, the fractional conversion model was applied to fit the isothermal-isobaric inactivation data [Eq. (2)]. More drastic conditions of temperature/pressure led to a fast inactivation period followed by a slower decay and as a result a biphasic model was applied to estimate the inactivation rate constants of the labile fraction [Eqs. (1)]. The inactivation rate constants for the labile fraction of purified pepper PME (pH 5.6) were estimated by non-linear regression analysis (Proc NLIN, SAS), according to the most adequate inactivation model (Table 1). For pressures higher than 300 MPa, a synergistic effect of pressure and temperature can be noticed. But in the high-temperature ($\geq 54°C$) and "low"-pressure ($P \leq 300 MPa$) region, on the contrary, an antagonistic effect of pressure and temperature was observed. In this range, a pressure increase resulted in a decrease of the inactivation rate constant.

The Arrhenius relation was valid in the whole pressure domain studied, and therefore, the activation energy could be determined by linear regression analysis of equation 3. According to Table 1, an increase of pressure resulted in a decrease of the E_a. A linear relation between the natural logarithm of E_a and pressure could be could be established ($R^2 = 0.91$). In the high-pressure region (≥ 600 MPa), a value of 43 $kJmol^{-1}$ (average) was found, which is similar to commercial orange PME in distilled water (~ 60-70 $kJmol^{-1}$) and purified orange at pH 3.7 (~ 25-38 $kJmol^{-1}$) (Van den Broeck et al., 2000b).

Due to the observed antagonistic effect of pressure and temperature, the Eyring relation was not valid for the entire pressure domain. So, the estimation of V_a was restricted to the higher pressure region ($\geq 300 MPa$) and determined by linear regression analysis of equation 4. At temperatures below 54°C, V_a-values were between -35 and -27 cm^3mol^{-1} (Table 1), and no real trend was observed between V_a-values and temperature. At temperatures where at atmospheric pressure inactivation occurs ($\geq 54°C$), the pressure sensitivity of the inactivation rate constants was significantly reduced (~ -19 cm^3mol^{-1}). When compared to other PME sources, purified pepper PME seemed to be less pressure sensitive than carrot PME (Tris-HCL buffer, pH 7.0; -32.1 ± 3.7 cm^3mol^{-1}) but more than orange PME (citrate buffer, 3.7; -13.77 ± 2.92 cm^3mol^{-1}) at temperatures at which at atmospheric pressure inactivation occurs (Van den Broeck et al., 2000b; Ly Nguyen et al., 2003).

3.2 Activity studies of purified pepper PME

The pressure range studied varied from 0.1-600 MPa, while temperature range was from 35-60°C. The results are illustrated in Figure 1. At pH 5.6 and atmospheric pressure, the optimal temperature for activity of purified pepper PME was found within the range of 50-55°C. For commercial tomato PME, an optimum temperature of 55°C within the pH range of 7.0 to 7.5 was also observed (Van den Broeck et al., 2000a), while purified tomato PME presented 45°C and 35°C as an optimum temperature at pH 8.0 and 4.4, respectively (Verlent et al., 2004). The optimal temperature range for purified pepper PME activity at elevated pressures was broader (50-60°C) when compared to atmospheric pressure. A shift in the optimal temperature at elevated pressures towards elevated values has been observed for commercial and purified tomato PME (Van den Broeck et al., 2000a; Verlent et al., 2003).

At 200 MPa, a higher enzymatic activity of purified pepper PME was observed in the temperature domain of 50-60°C, as compared to atmospheric pressure; whereas at 300 MPa, a higher activity was only noted at temperatures above 55°C. As a consequence of this pattern (e.g., an increase in temperature and acceleration of the reaction under pressure), the catalytic activity of purified pepper PME at temperatures above 55°C is significantly greater at pressures up to 300 MPa as compared to atmospheric pressure.
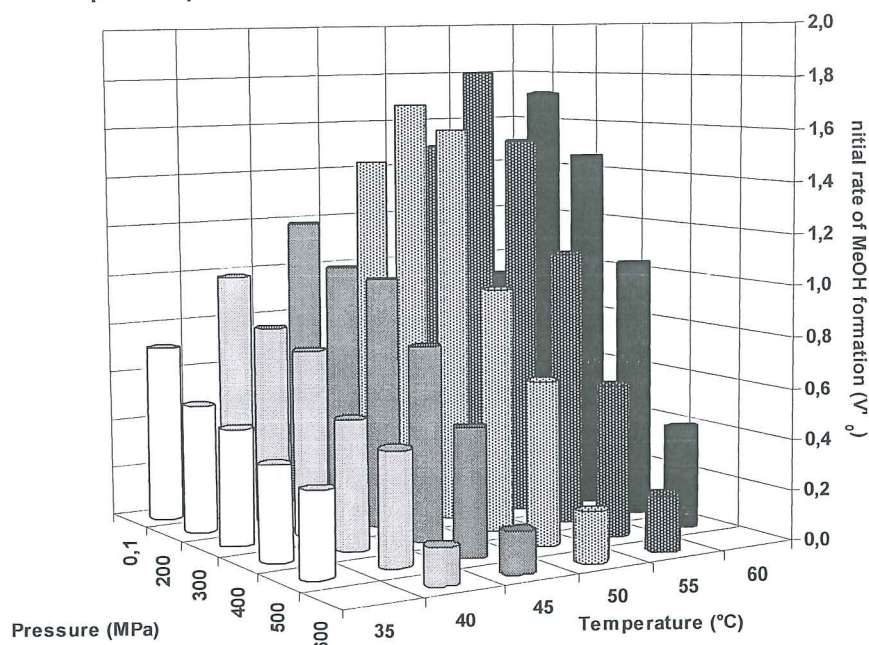
Figure 1 Initial rates of pepper-PME-catalysed methanol formation $\left(V_0'\right)$ as a function of temperature and pressure.

For temperatures lower than the optimal temperature (i.e., T<50°C), purified pepper PME activity decreased with increasing pressure. But for temperatures higher than 50°C, there was an enhancement of the pressure effect up to 200-300 MPa, followed by a decrease for higher pressures. Our findings seemed to be in agreement with Verlent and co-workers (2004). Purified tomato PME demonstrated a clear enhancing effect of pressure on the enzyme activity under the thermal/high-pressure conditions studied (35-65°C, 150-600MPa) when compared to atmospheric pressure, at pH 4.4 and 8.0.

Table 2 Pressure and temperature dependence of initial rates of purified pepper-PME-catalysed methanol formation $\left(V_0'\right)$ at pH 5.6.

P (MPa)	E_a (kJmol^{-1})	R^2		T (°C)	V_a (cm^{3}mol^{-1})	R^2
0.1	46.45±2.42[a]	0.99		35	3.93±0.19[a]	0.99
200	58.20±4.14	0.98		40	5.54±1.01	0.94
300	66.86±2.24	1.00		45	6.45±1.76	0.87
400	64.61±6.09	0.97		50	9.01±1.77	0.93
500	34.95±4.08	0.97		55	17.59±2.97	0.95
600	26.30±2.41	0.98		60	19.11±2.29	0.97

[a] Standard error regression.

The temperature dependence of V_0' at a given pressure (E_a) in the temperature range, in which the reaction is accelerated, could be described by Arrhenius equation [Eq. (5)]. The temperature sensitivity of V_0' seemed to be almost unaffected by pressure up 400 MPa (Table 2). From figure 1, it was possible to distinguish two different regions concerning the pressure dependence. Therefore, the V_a-values were estimated according to equation 6, in the pressure-temperature domain where V_0' decreases. Since the V_a-values were positive, pressure retards this enzymatic conversion (Table 2). The V_a-values slightly increased with increasing temperature up to 55°C; consequently, the pressure sensitivity of V_0' slightly increased with increasing temperature.

4. Conclusions

The obtained results clearly illustrate the potential of combined high-pressure/temperature treatments to modify the stability and activity of PME and create the opportunity to use high-pressure technology as a tool for improving textural properties of fruits and vegetables.

5. References

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